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http://dx.doi.org/10.1289/ehp.1206064

Online 11 June 2013

NIH National Institute of Environmental Health Sciences

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Keywords: CC10; CC16; lung development; *Macaca mulatta*; mucin; prenatal; SCGB1A1

Running Title: BPA alters lung secretory product expression

Acknowledgements: We thank the following people for their skilled technical assistance: Patricia Edwards, Dana Hill and Lisa Dillard. We thank Dr. Charles Plopper for assistance with Table 1 and Raymond Wong for his statistical expertise and analytical contribution to this manuscript. Funded by NIH grants ES016770, ES016249, OD01107/RR00169 and R21 ES021600. Lung sample processing methods development supported by P01 ES00628.

Competing financial interest declaration: The authors, with the exception of Dr. Van Winkle, declare no competing financial interests. Dr. Van Winkle receives grant funding from the American Petroleum Institute (API) to study naphthalene and has received honoraria from API to speak at conferences on naphthalene.

Abbreviations:

AHR (airways hyperresponsiveness), BPA (Bisphenol A), CCSP (Clara cell secretory protein), DGA (days gestation age), NHANES (National Health and Nutrition Examination Survey), qRT-PCR (quantitative Real time polymerase chain reaction), SEM (standard error of the mean)

ABSTRACT

Background: Bisphenol A (BPA) exposure early in life results in organizational changes in reproductive organs, but the effect of BPA on conducting airway cellular maturation has not been studied. Late gestation is characterized by active differentiation of secretory cells in the lung epithelium.

Objectives: We hypothesized that BPA exposure disrupts epithelial secretory cell development in the fetal conducting airway of the Rhesus macaque.

Methods: We exposed animals to BPA during either the second (early term) or the third trimester (late term). There were 4 treatment groups 1) Sham control early term, 2) Sham control late term, 3) BPA early term (BPA-early) and 4) BPA late term (BPA-late). Because cellular maturation occurs non-uniformly in the lung, mRNA and protein expression was defined by airway level using microdissection.

Results: BPA exposure of the dam during late term significantly accelerated secretory cell maturation in the proximal airways of the fetus; both Clara cell secretory protein (CCSP) and MUC5AC/5B mRNA and protein expression increased.

Conclusions: BPA exposure during late gestation accelerates secretory cell maturation in the proximal conducting airways. We have identified a critical window of fetal susceptibility for BPA effects on lung epithelial cell maturation in the third trimester. This is of environmental health importance because increases in airway mucins are hallmarks of a number of childhood lung diseases that may be impacted by BPA exposure.

INTRODUCTION

The respiratory health effects of bisphenol A (BPA) have been of recent interest (Kwak et al. 2009; Midoro-Horiuti et al. 2010; Nakajima et al. 2012; Roy et al. 2012). BPA is an organic chemical used in the production of polycarbonate plastics and epoxy resins. These plastics are found in food and drink packaging and are used as lacquers in food cans and bottle tops. BPA can migrate into food from these containers (Carwile et al. 2009; Rudel et al. 2011) and is also found in indoor air and dust (Inoue et al. 2006; Wilson et al. 2007). Over 90% of US urine samples tested in the National Health and Nutrition Examination Survey (NHANES) have measurable levels of BPA (Calafat et al. 2008), indicating widespread and continual exposure. Exposure levels for adult humans are in the 0.3 – 22.3 ng/mL range for unconjugated BPA in serum (Padmanabhan et al. 2008; Vom Saal et al. 2007), although a recent study found that consumption of canned soup resulted in short term 1000 fold increases (Ohshima et al. 2007). Plasma BPA levels in pregnant women and in the fetus have a similar range (Schonfelder et al. 2002). In adults, BPA pharmacokinetics have been found to be similar between mice and monkeys with linear kinetics (Taylor et al. 2011) and fairly complete clearance, therefore high serum levels in adult humans reflect continual exposures.

There is concern that current levels of exposure to BPA may adversely impact human development. In a companion study to our current research, BPA accelerated prenatal development of the rhesus monkey mammary gland including increased mammary bud density and overall gland maturation, similar to what has been seen in rodent studies (Tharp et al. 2012; Vandenberg et al. 2007). In a mouse ovalbumin sensitization model, maternal exposure to BPA increased asthma hallmarks such as eosinophils in bronchoalveolar lavage fluid and airways

hyperresponsiveness in offspring (Midoro-Horiuti et al. 2010), although histology of the lung was not characterized. BPA is related to allergic sensitization in animal models and in humans (Chu et al. 2006; Midoro-Horiuti et al. 2010; Ohshima et al. 2007), although lung effects have been little studied.

Many human lung diseases are characterized by abnormal epithelial cell secretions, particularly of mucous. Within the conducting airways, both mucins and Clara cell secretory protein, CCSP, have roles in airway disease (Ramsay et al. 2001; Voynow 2002), mature during pre-and postnatal development and are among the most abundant secretory proteins in lung tissue. MUC5AC and MUC5B are the predominant secreted gel forming mucins (Evans et al. 2009) with MUC5AC at as much as 300 fold lower levels than MUC5B during fetal lung development. CCSP is thought to have a protective role in the airways, regulating immune responses and attenuating oxidant stress (Plopper et al. 2005; Snyder et al. 2010). In general mucin expression is more abundant in proximal airways and CCSP expression is more abundant in distal airways corresponding to the differential abundance of mucous cells and Clara cells, respectively, in these airway regions. We selected CCSP and MUC5AC/B to study because these secretory proteins mature during the time periods spanned by this fetal BPA exposure. Further, the rhesus monkey lung is an excellent model for human fetal lung development in that it recapitulates the cellular and anatomic composition, as well as the timing (see Table 1), of human lung development (Plopper and Hyde 2008). In contrast, rodent models have airway secretory cells that are relatively immature at birth and do not contain mucous goblet cells throughout the tracheobronchial tree as the primary secretory cell type.

Lung epithelial development occurs in a series of highly choreographed sequences of events that span the pre and postnatal period (Plopper and Fannuchi 2004). Proximal conducting airway

epithelial cells mature earlier than distal airways. Because prenatal lung development is site specific in the conducting airways, and the late fetal time period is one of dynamic change, we have incorporated site specific methods into our analysis of conducting airway gene and protein expression. Exposure to toxicants during the prenatal period that disturb the normal course of development can result in disease later in life. The incidence of asthma is escalating in children and there is a hypothesis that environmental factors may be related to the increasing incidence. Interestingly, as pointed out by (Midoro-Horiuti et al. 2010), this rise in asthma prevalence (Vollmer et al. 1998) began 20 years after the widespread use of plastics began in the 1950s.

The effect of BPA on lung maturation in an animal model with cellular structure and airway architecture similar to humans, such as the rhesus monkey, has not been studied. The goal of the current study is to address three key issues: 1) to define the normal pattern of expression of airway secretory proteins (CCSP, mucins) in the fetal rhesus monkey lung, 2) to determine if prenatal exposure to an environmentally relevant level of BPA changes the abundance of these key secretory proteins and 3) to determine if there is a window of susceptibility for BPA effects on prenatal lung development.

METHODS

Animals: Adult female rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center as previously described (Hunt et al. 2012)(see Animals, Supplemental Material). Animal protocols were approved by the Animal Care and Use Committee of the University of California, Davis; all studies were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were treated humanely and with regard for alleviation of suffering.

Only females with a history of normal menstrual cycles were selected for this study (ages from 6 to 13 years). Animals were naturally mated. Pregnancy was detected by ultrasound examination and estimated day of conception (gestation day - GD 0) was assigned. At approximately GD 40, the sex of all fetuses was determined and only those with female fetuses continued in this study as the originating project for these samples was designed to study BPA effects on oogenesis. This study is part of a series of studies whose primary goal is to assess the effects of BPA on organogenesis in nonhuman primates using a dose that results in serum levels of BPA similar to those found in humans. Due to the expense of these studies, several laboratories shared tissues derived from the parent study. Tissues were obtained at 100 and 150 days gestational age (Campos-Bedolla et al. 2008) to study effects in the second and third trimester, respectively (Figure 1A).

BPA Dosing: Deuterated BPA (dBPA, CDN Isotopes, Quebec, Canada) was used in this study because it can be clearly distinguished by isotope dilution liquid chromatography-mass spectrometry (LC-MS), thus eliminating concern about potential BPA contamination from materials used in the preparation, handling or shipment of samples. dBPA in the dams was delivered in biocompatible silastic tubing implants placed subcutaneously via trocar in the scapular region so that the animals were continually exposed to BPA. Treatment days were at either mid-gestation (early dosing), GD 50 to 100; or late gestation (late dosing), GD 100 to 150 (see Figure 1A). Term is approximately GD 165. Implants were removed and replaced with freshly prepared implants after about 25 days of treatment (half way through the dosing period) to assure that BPA levels remained near the maximum release rate. Silastic tubing implants for each animal were prepared as previously described (Hunt et al. 2012). The calculated release rate of 1.056 mg/24 hours was based on test capsules loaded with ^{3}H-BPA that were placed in

saline solution for up to 40 days. The resulting serum levels for non-pregnant test animals that received implanted capsules for 2 weeks ranged from 2.2 to 3.3 ng/mL unconjuated dBPA, within the range (0.5-22.3 ng/mL) measured in humans (Padmanabhan et al. 2008). Age matched control animals were treated with sham, corn oil implants (N= 2 of each age). Additional lobes prepared similarly to those for qRT-PCR analysis in this study were available from age matched sham control animals given corn-oil treated fruit (N=4-6 of each age). Control animals from the two studies did not vary from each other and so were pooled for at each age for qRT-PCR analysis (N=7-8). The *in vivo* portion of the study was conducted with only two control animals assigned to each gestation age group (early and late) due to limited pregnant dam availability. We attempted to compensate for the small N by pooling current and historical control data. However, the particular protocol for inflation with fixative and site specific localization of airways used in the current study for histologic sample preparation differed significantly from historical control sample processing making additional lobes from historic controls inappropriate for comparable histologic and immunohistochemical staining and subsequent morphometric analysis, resulting in N=2 for each control group/age for these endpoints. BPA treated samples were N=6 for each age.

Lung tissue processing: All fetuses were removed by cesarean section at gestational Day 100 for the early dosing group and gestational Day 150 for the late dosing group. The lobes of the lung were subdivided and processed as described in the online supplement. Because lung maturation occurs in a proximal to distal direction, two groups of airway generations were analyzed (see Figure 1B) for qRT-PCR, high-resolution histopathology and immunohistochemistry: proximal airways (generations 3-4, intrapulmonary bronchi) and distal airways (airway generations 8-10, distal bronchioles)(for additional details, see Lung Processing in Supplemental Material).

Immunohistochemistry and histochemistry: Paraffin sections from 2 control animals and 4 treated animals per age (~3-4 slides/animal) were immunostained for Clara cell secretory protein (Bio Vendor, 1:2000). Controls included the substitution of primary antibody with phosphate buffered saline, which resulted in loss of specific staining (see Supplemental Material, Figure S1). Mucous cells were stained with Alcian Blue-Periodic Acid Schiff histologic stain following manufacturer's instructions (American MasterTech, Lodi, CA)(Caramori et al. 2009)(for additional details, see Immunohistochemistry and histochemistry in Supplemental Material).

Morphometric histopathology: Because the amount of site specific paraffin sections was limited in these fetal lungs, we only quantified the abundance of mucosubstance in the airway epithelium of proximal (intrapulmonary generations 1-3) conducting airways was determined using stereologic assessment of lung structure (Hsia et al. 2010). Paraffin sections (5 um thick) from 2-4 animals/group/age (2-4 slides/animal) were stained for mucin using Alcian Blue/PAS staining (Caramori et al. 2009). The volume fraction and mass of mucosubstance in the proximal epithelium as well as epithelial thickness were assessed in 2 controls and 4 BPA treated animals per age (for additional details, see Morphometric histopathology in Supplemental Material).

Gene Expression: CCSP, *Muc5AC*, and *Muc5B* gene expression was measured using real time reverse transcriptase polymerase chain reaction (qRT-PCR)(N=5-8)(for additional details, see Gene Expression in Supplemental Material).

Statistics: Fold change of gene expression in microdissected airways from 5-8 animals per time point was calculated using the comparative Ct ($2^{-\Delta\Delta CT}$) method as described previously by Applied Biosystems (Livak and Schmittgen 2001). Results were reported as fold changes relative to proximal late control and graphed as means +/- SEM. Statistical outliers were eliminated

using the extreme studentized deviate method (Graphpad, La Jolla, CA). Undetected and samples observed below detection limit were treated as nondetects, and values were imputed using the natural-log regression on order statistics (lnROS) method (Helsel 2005; Shumway et al. 2002) using ProUCL (U.S. EPA, Atlanta, GA). Multivariate analysis of variance (MANOVA) was applied against age, compartment and exposure factors when appropriate. Pair-wise comparisons were performed individually using a one-way ANOVA followed by PLSD post hoc analysis using StatView (SAS, Cary, NC). P values of ≤ 0.05 were considered statistically significant. Morphometric analysis of proximal airway mucosubstance was assessed in control (N=2) and BPA exposed (N=3-4) animals. Due to the small number of control animals (N<3), there were not enough data to conduct rigorous statistical inferences between groups. Only descriptive statistics (arithmetic mean) are presented.

RESULTS

Normal expression of secretory products during prenatal development (Figure 2): *Muc5AC* did not vary significantly by age or airway level (distal early vs. late P=0.07), although a majority of samples (15 of 27) tested in distal airways at 100 DGA did not have detectable message for this gene (Figure 2A). *Muc5B* mRNA did not differ significantly with age or compartment but was slightly more abundant in proximal airways vs. distal airways late in gestation, at 150 DGA (Figure 2B). *CCSP* mRNA was significantly more abundant in distal bronchiolar airways (generations 8-10)(Figure 1B) at 150 DGA (P=0.002) than in proximal airways or in airways earlier in gestation (P=0.001)(Figure 2C). Maturation of the airway epithelium over the time period of this study was apparent on high resolution resin sections. Glycogen, present as clear cytoplasmic inclusions in the tall pseudostratified epithelium, was more abundant at 100 DGA

and the basement membrane was less marked at 100 DGA than at 150 DGA (compare Figure 2D with 2E). Mucous cells appear more mature at 150 DGA, with a protruding apex and a cytoplasm containing granules (Figure 2E).

Expression of secretory products following exposure to BPA: The mRNA for *CCSP* was detected in all ages and in both proximal (Figure 3A) and distal (Figure 3B) airways. BPA exposure in late gestation resulted in an insignificant increase in *CCSP* mRNA expression in the proximal bronchi vs. control (P=0.2)(Figure 3A). Early gestation CCSP gene expression was unaffected by BPA in proximal or distal airways. Distal airways at 150 DGA contained significantly more *CCSP* mRNA expression than proximal (P=0.002) or earlier 100 DGA (P=0.001) airway levels (see Figure 3A, 3B). CCSP protein was localized to both tall pseudostratified epithelial cells of the large airways as well as simple cuboidal epithelium lacking cilia in the distal airways (Figures 3C-F). BPA exposure markedly increased the distribution and abundance of CCSP protein in the airway epithelium (Figure 3D and F).

Muc5AC mRNA levels were changed by airway level and BPA exposure (Figure 4A and B). Similar to effects on *CCSP* seen at 150 DGA, late gestational exposure elicited no significant effect in proximal (P=0.2) airways but resulted in a significant decrease in distal (P=0.02) airway mRNA expression. However, expression of *Muc5B* in the proximal bronchi was significantly increased in the BPA-late group approximately 6 fold compared to 150 DGA control animals (P=0.005) and distal BPA-150 DGA airway expression (P=0.003)(Figure 4C and D). Distal bronchiolar expression of *Muc5B* was not changed by age or BPA exposure.

Mucins detected using AB/PAS histologic staining indicated mucosubstance positive cells in proximal airway epithelia. Morphometric assessment of Volume fraction, *Vv* (Figure 4E) and

Volume per surface area or mass, *Vs* (Figure 4F) of mucosubstance positive cells and proximal epithelial thickness (*t*), um (Figure 4G), showed that all three parameters increase with age. BPA enhances this trend by increasing volume fraction in both early and late gestation (Figure 4E) but only increased mass in the BPA-early group (Figure 4F). BPA late in gestation reduced epithelial thickness compared to matched controls to levels just above the early groups. Figure 5 shows increased incidence of mucosubstance positive cells in the proximal airways of BPA exposed animals compared to controls during both late (compare Figure 5A with Figure 5B) and early (compare Figure 5C with Figure 5D) gestation.

DISCUSSION

Our data indicate that exposure to environmentally relevant levels of BPA during fetal lung development can alter expression of secretory genes (*Muc5B, CCSP*) and proteins (MUC5B and CCSP) in the conducting airways. Further, we found that this increase is most pronounced in the proximal conducting airways, bronchi. BPA exposure later in gestation (roughly spanning the 3rd trimester) has a greater effect on epithelial secretory maturation than an earlier exposure. Thus we have identified a "critical window" of timing in development for BPA alteration of the normal lung. It is likely that this critical window of time will also apply to human exposures in the third trimester as the timing of cellular development, as well as conducting airway architecture/cellular composition, in the rhesus monkey lung closely recapitulates that in humans (Table 1) (Tarantal and Gargosky 1995; Burri1997; Plopper and Hyde 2008). Our results also underscore the importance and feasibility of using site specific methods to study fetal development in the rhesus monkey; because cell maturation in the conducting airways occurs in

a proximal to distal direction, comparing like sites is important due to the large gradient in differentiation between different airway generations.

There is a dichotomy in BPA's effects on conducting airway mucins: MUC5B is affected by exposure and MUC5AC expression is not. Mucins are critical for maintenance of normal lung homeostasis. They contribute to the liquid lining layer of the airways and assist with removal of foreign substances and regulation of inflammation. Overly abundant secretion and storage of mucous can cause airway obstruction found in a number of lung diseases including asthma and bronchitis. BPA exposure increases both mucous cell abundance and *Muc5B* gene expression. BPA increases the percentage of mucous cells (*Vv,* Figure 4E) but the mass of mucous cells (*Vs*; Figure 4F) is only increased in the early exposure group. This is possibly due to decreased epithelial thickness (*t*; Figure 4G) in the BPA late exposure group where the mucous cells make up a higher percentage of a thinner epithelium. BPA effects on *Muc5B* may be due to the binding of the parent or its metabolites to estrogen receptors (Okuda et al. 2011). Estrogen (17 beta- estradiol) is known to induce *Muc5B* expression in airway epithelial cells (Choi et al. 2009) via ER-alpha. BPA interacts with both nuclear estrogen receptors that regulate transcription as well as cell membrane bound estrogen receptors (Vom Saal et al. 2007). MUC5B is found in both airway submucosal glands and in surface epithelial goblet cells (Finkbeiner et al. 2011). MUC5AC is more predominant in the surface goblet cells (Finkbeiner et al. 2011). We were not able to analyze the effects of BPA on glandular development; we had too little sample to define this histologically and the airway microdissection method we used for qRT-PCR combines both airway glands and the surface epithelium in the same sample. Future studies could correlate glandular vs. airway epithelial expression of MUC5B using laser capture microdissection, as has been done in study of salivary glands in humans (Kouznetsova et al. 2010). Our data shows that

BPA exposure increases the expression of both the gene and the protein for the two most abundant secretory proteins, MUC5B and CCSP, in the airways. Increased expression is apparent with more cells containing the protein, increased abundance of the protein per cell and increased gene expression on an airway basis.

The biologic relevance of the increase in CCSP in the proximal airways is unknown. There is little data showing effects of increased CCSP. However, decreased secretion of CCSP has been found in the lavage fluid of patients with asthma (Van Vyve et al. 1995) and polymorphisms in this gene that confer low serum levels of CCSP correlate with an increased risk of asthma in children with allergic rhinitis (Ku et al. 2011). In general, CCSP is considered a beneficial protein, so much so that rhCCSP has been considered as a therapy in infants with respiratory distress (Abdel-Latif and Osborn 2011). CCSP has not been reported to be responsive to estrogens in the lung but can be increased by exposure to interferon-gamma (Ramsay et al. 2003) and TNF-alpha (Cowan et al. 2000). The reason CCSP is upregulated by BPA in the epithelium of the large airways of fetal rhesus monkeys will require further investigation.

It is not known whether increased expression of MUC5B and CCSP is an aberrant process that could persist and lead to pathology/disease later in life or whether this is actually a neutral or even beneficial process. This is a limitation of the current study which does not contain a follow up period succeeding exposure to determine if these changes are persistent. What makes the current findings worrisome, however, are previous studies that demonstrate fetal BPA exposure increases allergic sensitization and asthma hallmarks in mouse models (Midoro-Horiuti et al. 2010; Nakajima et al. 2012). In the mouse, when BPA exposure spanned the period from before implantation to weaning, BPA exposure accelerated airways hyperresponsiveness (AHR) to allergen challenge and increased eosinophils in the lavage fluid in the offspring of BPA exposed

dams (Midoro-Horiuti et al. 2010). A follow up study also found AHR following a shorter BPA exposure period that only included the prenatal period, from pre-implantation to birth (Nakajima et al. 2012). Our exposure paradigm is a still shorter period, spanning most of a trimester late in gestation, yet still shows significant effects on the lung. BPA also affects the immune system, leading to speculation that BPA may be involved in the development of asthma and allergy (Kwak et al. 2009). BPA has been shown to increase IL-4 production in primed CD4+ T cells and also increases antigen specific IgE in primed mice, potentially enhancing allergic responses (Lee et al. 2003). BPA exposure has also been found to alter innate immunity slightly in mice exposed to influenza (Roy et al. 2012). If mucous cell abundance is increased by BPA, and AHR/allergy also is increased, this could synergize and increase airway obstruction, making asthma more severe. Future studies of BPA effects on lung cellular development and asthma are needed and should focus on exposures that encompass this late fetal time period and also include prolonged follow up to determine long term effects of early life exposure to BPA.

The current study found significant effects in the fetus when the dam was exposed. Significant effects of chemical exposure during prenatal development occur for many reasons: a critical window of susceptibility, enhanced delivered dose to the fetus due to differences in fetal-maternal detoxification/metabolism, formation of unique metabolites or selective uptake by compartments unique to the fetus including amniotic fluid or placenta. The amniotic fluid chemical composition should be investigated as it bathes the epithelium lining the fetal lung and so increases in chemical concentration in this compartment would affect the epithelium directly. It is unknown whether all or some of these factors contribute to BPA's fetal effects on the lung in this model. Certainly the chemical composition of the amniotic fluid should be considered as this fluid bathes the epithelium lining the lung which is affected by prenatal BPA exposure. The

lung contains substantial xenobiotic metabolizing enzymes that can contribute to the local burden of metabolites and have transient expression during lung development. This is of interest as some BPA metabolites have more estrogenic activity than the parent molecule (Nakamura et al. 2011). Cytochrome P450 monooxygenases mature late in development and localize to the epithelial lining layer of the respiratory tract, the very area that this study shows is affected by fetal BPA exposure. BPA is metabolized by cytochrome P450s and is detoxified through glucoronidation and sulfation. The balance of activation and detoxification is likely an important determinant of BPA effects and this includes both maternal and fetal capabilities. Studies are needed to define the relative role of these enzymes and their influence on pharmacokinetics in the prenatal period, particularly during the third trimester in primates.

It is important to acknowledge the limitations of this study. The N is small for the histologic endpoints, and, although the N is larger for the significant gene expression data, replication and extension of the study would provide more confidence in the study results. There is a lack of an exposure group that is followed into the postnatal period which would allow for assessment of persistence of effect as well as study of pulmonary function and lung compliance. Finally the monkeys in this study are all female fetus' because the original study was designed to look at effects on oogenesis. Future studies should include both sexes as asthma is more prevalent in males prior to puberty (Vink et al. 2010).

CONCLUSIONS

We conclude that BPA exposure during late gestation accelerates secretory cell maturation in the proximal conducting airways. We have identified a critical window of fetal susceptibility for BPA effects on lung epithelial cell maturation in the third trimester of a highly relevant model,

the rhesus monkey. This is of environmental health importance because increases in airway mucins are hallmarks of a number of childhood lung diseases that may be impacted by BPA exposure.

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Table 1: Comparison of stages of fetal development in Rhesus macaques and humans[a]

Stage of Pregnancy			Stage of Lung Development		
	Gestational age (Days)			Gestational age (Days)	
Trimester	Macaque	Human	Lung Stage	Macaque	Human
1st	to 55	to 90	embryonic	21-55	
2nd	55-110	90-180	pseudoglandular	56-80	42-112
			canalicular	80-130	112-168
3rd	110-165	180-270	saccular	131-165	168-270

[a]Based on information in the following publications: (Burri 1997; Plopper and Fannuchi 2004; Tarantal and Gargosky 1995)

FIGURE LEGENDS

Figure 1: Timeline of BPA exposure (A) and the sampling scheme for microdissected airways in the lung (B). This study assessed two time periods of BPA exposure in the fetus; an early exposure that ended on gestation day 100 (second trimester) and a late exposure that ended on gestational day 150 (third trimester). Age matched sham treated control animals were included allowing analysis of normal fetal development as well as BPA effects. Exposure groups were: 1) Sham control early term, 2) BPA-Early, 3) Sham control late term and 4) BPA-Late. The lung diagram (B) illustrates the two airway sites sampled including intrapulmonary proximal bronchi generations 3-4 and distal airway generations 8 through respiratory bronchioles.

Figure 2: *Expression of secretory products and epithelial morphology during normal prenatal development.* Expression of secretory product mRNA in microdissected proximal airways and distal airways (A-C) as measured by qRT-PCR and reported as fold change compared to late control proximal airway. *Muc5* mRNA expression did not vary significantly but had slight, nonsignificant increases in *Muc5AC* (A) in distal airways (P=0.07), and *Muc5B* (B) in proximal airways late in gestation compared to both early proximal (P=0.4) and late distal compartments (P=0.2). C. Clara cell secretory protein (*CCSP*) gene expression is significantly increased late in gestation in distal airways in comparison to early distal (P=0.001) and late proximal (P=0.002) ages. $P<0.05$ * = significantly greater than same compartment, early age expression, # = significantly greater than same age, proximal compartment expression. N= 5-8 for qRT-PCR values represented as mean ± SE (one-way ANOVA and PLSD post hoc analysis). Representative high resolution histopathology of proximal airway epithelium in resin sections stained with methylene blue/azure II stain. D. Proximal airway epithelial cell morphology early

in gestation (D100). E. Proximal airway epithelial cell morphology late in gestation (D150). M indicates mucous cell. Bar = 50 um.

Figure 3: *Effect of exposure to bisphenol A (BPA) on expression of Clara cell secretory protein (CCSP).* Expression of *CCSP* mRNA in microdissected proximal (A) and distal (B) airways as measured by qRT-PCR and reported as fold change compared to late control proximal airway. Distal control (B) mRNA expression increased significantly with age (P=0.001) and late distal control was also significantly greater than the age matched proximal (A) control (P=0.002). P<0.05 * significantly greater than same compartment, early age expression, # = significantly greater than same age, proximal compartment expression. N = 5-7 for qRT-PCR values, represented as mean ± SE (one-way ANOVA and PLSD post hoc analysis). Pattern of CCSP protein expression detected using immunohistochemistry on sections of proximal airways. Representative sections of late control (N=2) (C) and late BPA treated animals (N=4) (D) show a substantial increase in CCSP protein expression in columnar epithelial cells with similar morphologic characteristics to mucous cells. CCSP protein in distal airways of late control (E) and late BPA exposed (F) was expressed in cells resembling Clara cells (arrows). Magnification bar for C-F = 50 um. [E indicates epithelial cells.

Figure 4: *Effect of exposure to bisphenol A (BPA) on mucin expression*. *Muc5AC* (A and B) and *Muc5B* (C and D) gene expression was measured by qRT-PCR in microdissected proximal (A and C) and distal (B and D) airways. Gene expression changes are reported as fold change compared to late proximal control. Late proximal BPA (A) is significantly greater than matched distal (B) group (P=0.02). Late distal control *Muc5AC* expression (B) was significantly greater than matched BPA (P=0.02). Late proximal BPA *Muc5B* expression (C) was significantly greater than matched control (P=0.005) and age and treatment matched distal group (D)

(P=0.003). P<0.05 *= significantly greater than same compartment BPA treated group, # = significantly greater than same compartment control group, †= significantly greater than same age-treatment distal compartment. N= 5-8 for qRT-PCR values, represented as mean ± SE (one-way ANOVA and PLSD post hoc analysis). Morphometric assessment of Volume fraction, *Vv* (E), and Volume per surface area (mass), *Vs* (F), of mucosubstance positive proximal epithelial cells, and proximal epithelial thickness, um (G). Morphometric measurements of proximal airways are presented as individual data points (1/animal) with the bar representing the arithmetic mean (N=2-4). BPA increases the volume fraction of mucosubstance in proximal epithelia in both early and late gestation (E) and the mass (F) of mucosubstance (*Vs*) in early gestation. Epithelial thickness decreases with BPA treatment in late gestation (G).

Figure 5*: Effect of exposure to bisphenol A (BPA) on proximal epithelial mucosubstance expression.* Representative sections of airway epithelial mucosubstances, as detected using Alcian Blue/PAS staining in proximal airways of late control (A), late BPA (B), early control (C) and early BPA (D) exposed groups). Mucosubstance was localized to cells resembling goblet cells (arrows). Magnification bar = 50 um.

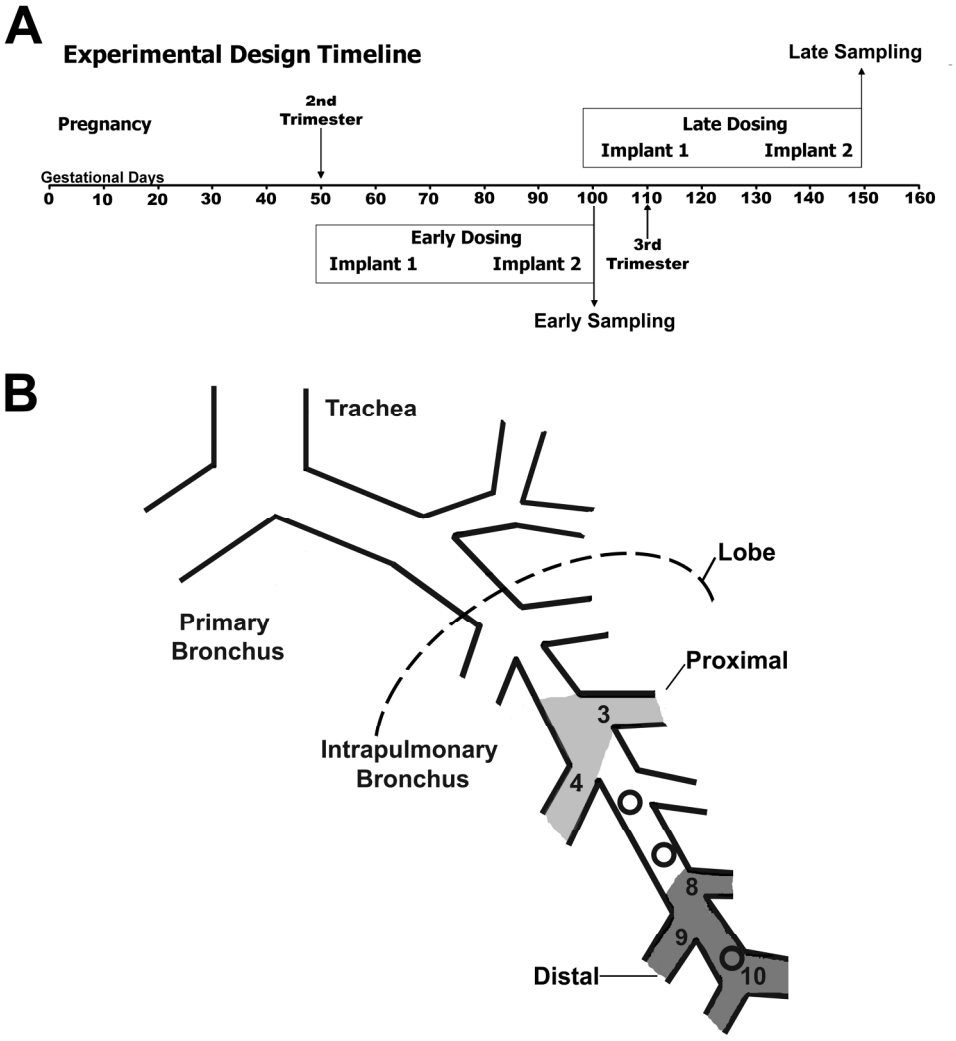

Figure 1
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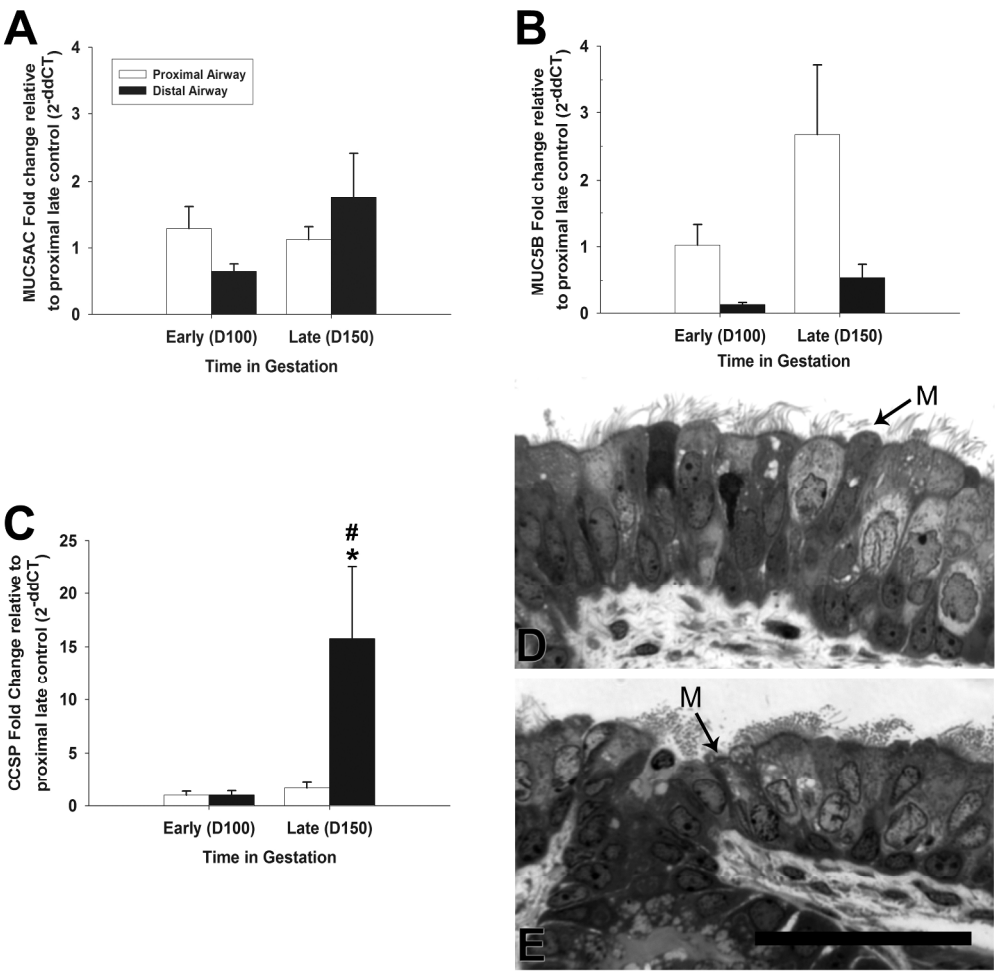

Figure 2

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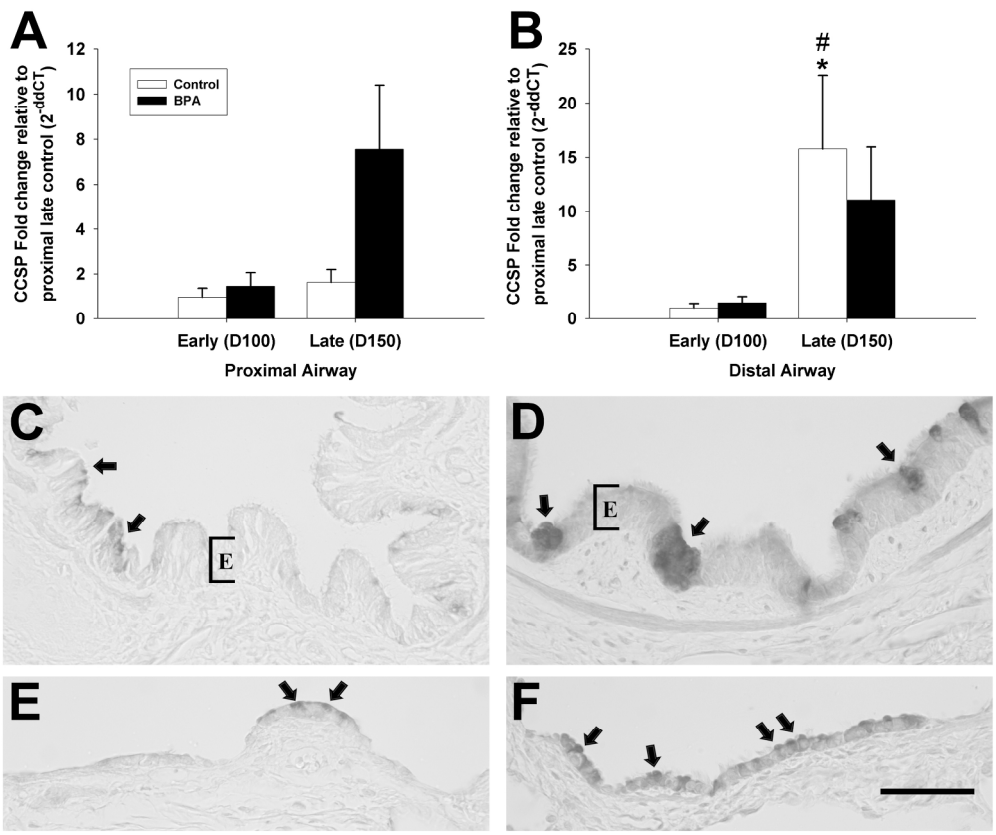

Figure 3

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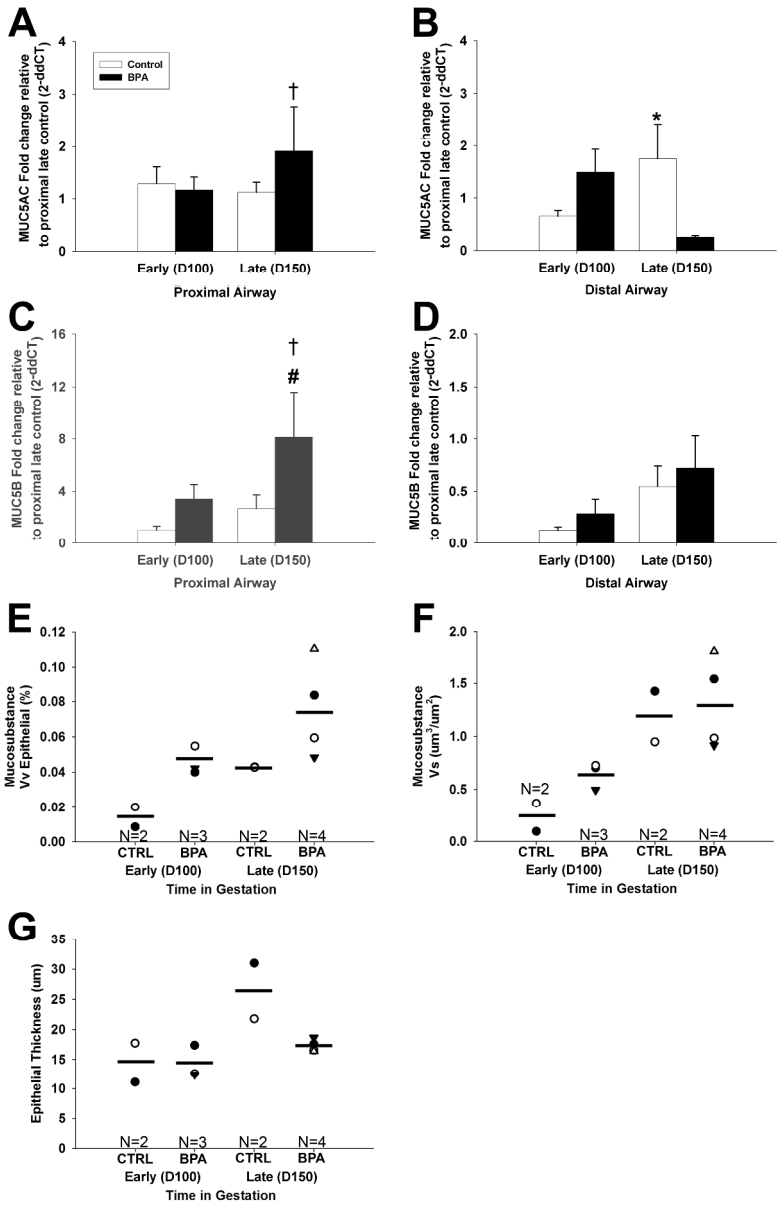

Figure 4

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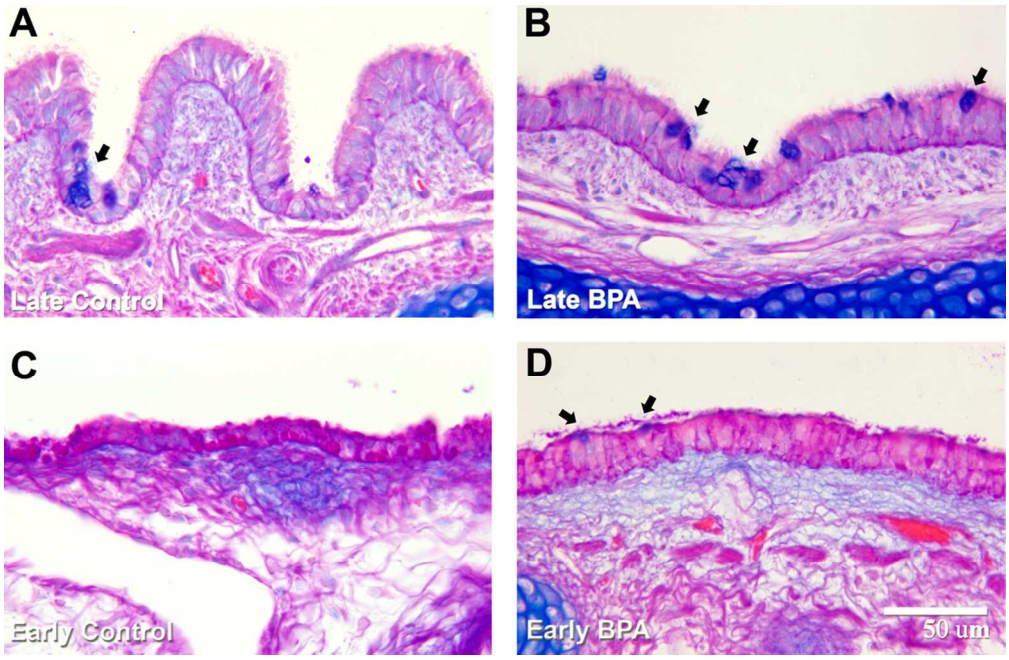

Figure 5
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